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Brucella suis Prevents Human Dendritic Cell Maturation and Antigen Presentation through Regulation of Tumor Necrosis Factor Alpha Secretion[▽]

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***Brucella* is a facultative intracellular pathogen and the etiological agent of brucellosis. In some cases, human brucellosis results in a persistent infection that may reactivate years after the initial exposure. The mechanisms by which the parasite evades clearance by the immune response to chronically infect its host are unknown. We recently demonstrated that dendritic cells (DCs), which are critical components of adaptive immunity, are highly susceptible to *Brucella* infection and are a preferential niche for the development of the bacteria. Here, we report that in contrast to several intracellular bacteria, *Brucella* prevented the infected DCs from engaging in their maturation process and impaired their capacities to present antigen to naïve T cells and to secrete interleukin-12. Moreover, *Brucella*-infected DCs failed to release tumor necrosis factor alpha (TNF-α), a defect involving the bacterial protein Omp25. Exogenous TNF-α addition to *Brucella*-infected DCs restored cell maturation and allowed them to present antigens. Two avirulent mutants of *B. suis*, *B. suis bvrR* and *B. suis omp25* mutants, which do not express the Omp25 protein, triggered TNF-α production upon DC invasion. Cells infected with these mutants subsequently matured and acquired the ability to present antigens, two properties which were dramatically impaired by addition of anti-TNF-α antibodies. In light of these data, we propose a model in which virulent *Brucella* alters the maturation and functions of DCs through Omp25-dependent control of TNF-α production. This model defines a specific evasion strategy of the bacteria by which they can escape the immune response to chronically infect their host.**

Brucella is a facultative intracellular α2-proteobacterium that induces chronic infections in a wide variety of mammals, including field ruminants, humans, and marine mammals. In addition to the attention received from its classification as a potential weapon for bioterrorism (41), this bacterium is principally known because of its ability to induce infectious abortion in domestic animals and because brucellosis is the most frequent anthropozoonosis (20). The three most infectious species in humans are *B. melitensis*, *B. abortus*, and *B. suis.* Infection occurs through inhalation of aerosols or ingestion of infected food. Following invasion of the lymphoid system, the bacteria develop within mononuclear phagocytes, and infected cells play a crucial role in the dissemination of the bacteria in specific locations of the body. Also known as Malta fever, human brucellosis consists of an acute infection, characterized by undulant fever and asthenia, which evolves in 30% of infected patients into a chronic phase with erratic recurrent fevers and localized infections, such as endocarditis, encephalitis, and spondylitis. Chronic brucellosis patients display a T helper 2 (Th2)-specific immune response (19, 43). In mice, which are not natural hosts for *Brucella* and display a certain resistance to infection, the protection is conferred by a Th1-oriented immune response depending on gamma interferon-producing $CD4^+$ T lymphocytes (2, 3, 46). Therefore, the ability of *Brucella* to chronically infect human hosts seems to be related to its capacity to avoid establishment of a protective Th1-specific response (7, 19, 43, 55).

For several years, our laboratory and others have studied the interaction of *Brucella* with macrophages and identified several virulence mechanisms implicated in the interaction of *Brucella* with the innate immune system (1, 9, 10, 18, 21, 25, 29, 30, 39). Nevertheless, due to reduced implication of macrophages in the initiation of a specific immune response, the macrophage infection model is not suitable when the adaptive immune response is considered in the context of the *Brucella*-host interaction. Myeloid dendritic cells (DCs) have naturally emerged as interesting models. DCs serve as sentinels for the immune system; they ingest pathogens at the site of infection and migrate to secondary lymphoid organs, where they present pathogen-derived antigens to naïve T lymphocytes, thus initiating the specific immune response. Pathogens could target DCs during early stages of infection as a way of disabling and evading host immune responses. In a previous publication, we showed for the first time that human DCs are highly permissive cellular hosts for *Brucella* (5). In this report, we analyze the consequences of *Brucella* infection for DC physiology, particularly for maturation processes and antigen presentation to naïve T cells. Relationships with some bacterial proteins were studied, and the importance of DC infection for *Brucella's* virulence strategy and the pathogenesis of human brucellosis is discussed.

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MATERIALS AND METHODS

Bacteria. The *Brucella* strains used in this study are mutants of *B. suis* 1330. The strain referred to as the wild type (WT) constitutively expresses a green fluorescent protein (GFP). Every bacterial strain mentioned here has been de-

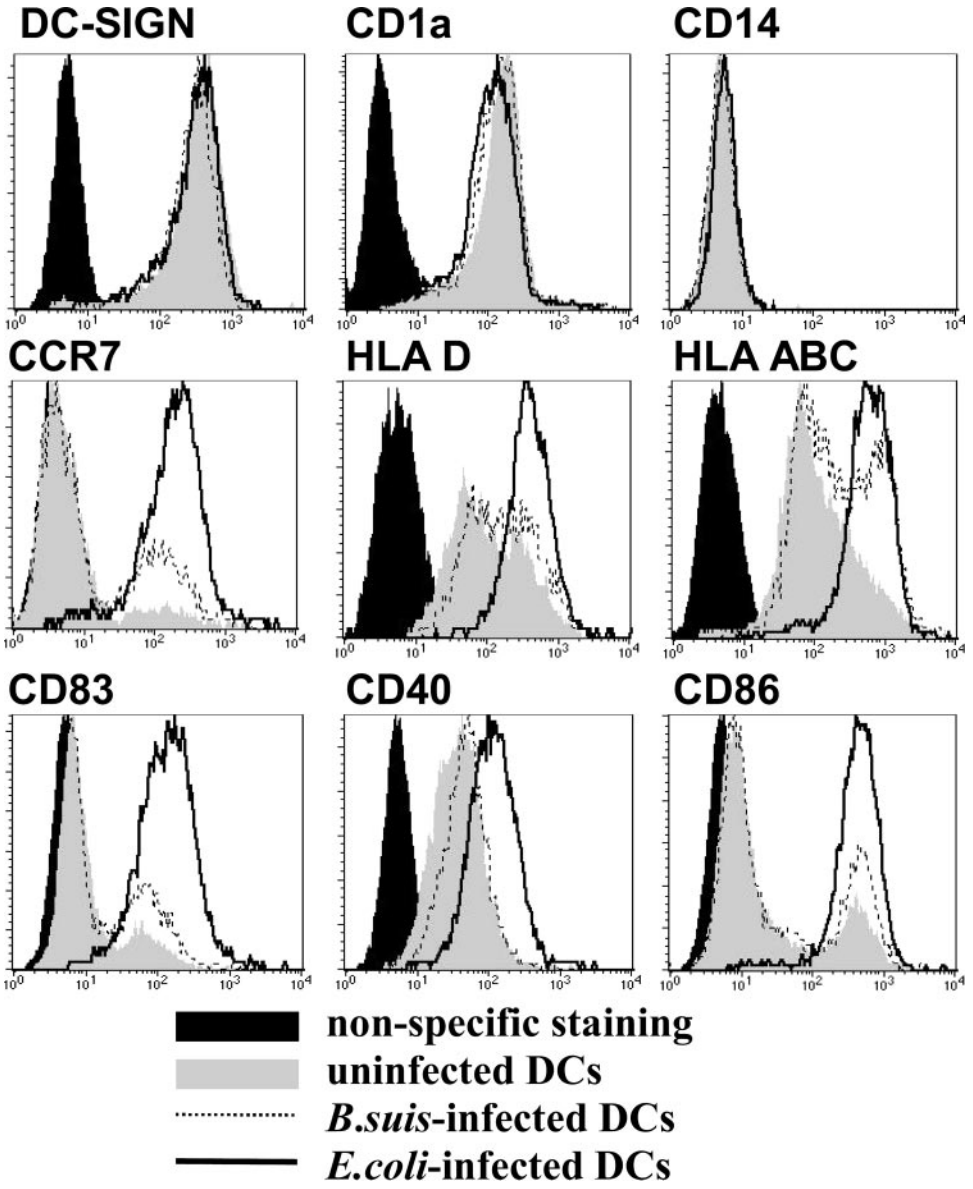

FIG. 1. Fluorescence-activated cell sorting analysis of DC maturation in response to infection by *B. suis*. Immature human DCs were infected with WT *B. suis* or *E. coli* for 48 h and then stained for maturation marker expression. Cytometry analysis histograms are presented for each surface molecule studied and show the results of 1 experiment that was representative of 22 independent experiments.

scribed in detail elsewhere (27, 30). Two *B. suis* mutants were used, in which the *bvrR* and *omp25* genes were inactivated: *B. suis bvrR (B. suis bcsp31*::GFP *bvrR*::mTn*5*Km2 [30]) and *B. suis omp25* (*B. suis* Δ*omp25*::*kan* [27]). The smooth character of the *Brucella* strains used in this study was originally controlled by crystal violet staining and immunoblotting techniques, as previously mentioned (26). No differences were observed between *B. suis* 1330 GFP and *B. suis* 1330 *bscp31*::GFP (parental strain of the *bvrR* mutant) (30), so we present data for only one control in the figures for clarity. The GFP-expressing strain *Escherichia coli* S17.1 D3 (49) was a generous gift from A. Givaudan, INRA UMR 1133, Montpellier, France.

Antibodies and reagents. The anti-human tumor necrosis factor alpha (TNF-α) antibody (R&D Systems, Minneapolis, MN) was used at a concentration of 2.5 μg/ml, and recombinant human TNF-α (rhTNF-α) (Immunotools, Friesoythe, Germany) was used at a concentration of 10 ng/ml.

Most antibodies used for DC phenotypic analysis were purchased from BD Pharmingen, San Diego, CA; the exceptions were mouse anti-CCR7 (R&D Systems) and anti-HLA-ABC (Beckmann-Coulter).

DC preparation. Immature DCs were prepared from peripheral blood circulating monocytes obtained by centrifugation on Ficoll-Hypaque (Sigma, Lyon, France) of buffy coat from healthy donors provided by the Etablissement Français du Sang. $CD14^+$ monocytes were purified by magnetic bead-positive separation (Miltenyi Biotec, Paris, France) and then differentiated for 5 days in complete medium (RPMI 1640, 10% fetal calf serum, 50 μM β-mercaptoethanol, 500 U/ml interleukin-4 [IL-4], 1,000 U/ml granulocyte-macrophage colony-stimulating factor [both cytokines were obtained from Immunotools]) (5).

Infection experiments. Immature DCs were harvested, resuspended in RPMI medium plus 10% fetal calf serum, and infected for 1 h at 37°C with bacteria at concentrations corresponding to a CFU/DC ratio of 5:1. The cells were then washed in phosphate-buffered saline (Invitrogen) and reincubated in fresh medium supplemented with 50 μg/ml gentamicin in order to kill remaining extracellular bacteria (21). In these experimental conditions, the rates of infection of DCs with *Brucella* and *E. coli* were similar, 29.6% ± 2.6% and 31.12% ± 3.2%, respectively. This was described in a previous study in which fluorescence microscopy experiments were performed by using GFP-labeled bacteria and by

counting viable intracellular bacteria (5). For assessment of intracellular proliferation, infected cells (2×10^5 cells/well) were washed and lysed at several times postinfection (p.i.) in 0.1% Triton X-100 (Sigma). The number of intracellular viable bacteria (in CFU per well) was determined by plating 10-fold serial dilutions on tryptic soy agar plates.

Maturation analysis. At 48 h p.i., DCs were labeled with mouse anti-human monoclonal antibodies followed by a phycoerythrin-conjugated goat anti-mouse polyclonal antibody (BD Pharmingen) and were analyzed with a FACSCalibur cytometer (Becton Dickinson, San Jose, CA).

Cytokine measurement. For cytokine measurement, supernatants were collected and concentrations of TNF-α and IL-12p70 were measured with an OptEIA human enzyme-linked immunosorbent assay set (BD Pharmingen) or by flow cytometry using a CBA Flex set (BD Biosciences).

Antigen presentation to naïve human T lymphocytes. Human naïve $CD4^+$ T cells were prepared using an EasySep human naïve $CD4^+$ T-cell enrichment kit (Stem Cell Technologies) according to the manufacturer's instructions. Naïve T cells ($CD3^+$ $CD4^+$ $CD45RA^+$) were stained intracellularly at 37°C in RPMI medium containing 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma-Aldrich), extensively washed in medium, and plated in a 96-well culture plate (10^5 cells per well). Infected DCs (24 h p.i.) were added at the required concentration so that the DC/T-cell ratios ranged from 0 to 0.1. Five days later, the cells were stained with a mouse anti-human CD3 antibody (UCHT1; BD Pharmingen), followed by an Alexa 647 $F(ab')_2$ fragment of goat anti-mouse immunoglobulin G (Molecular Probes, United Kingdom). Analysis was performed by flow cytometry using a FACSCalibur cytometer to detect the decrease in CFSE fluorescence intensity resulting from cellular divisions.

Statistical analysis. Wilcoxon rank tests or paired Student's *t* tests (in the case of normal distribution) were used to determine statistical differences, using the SigmaStat and R software.

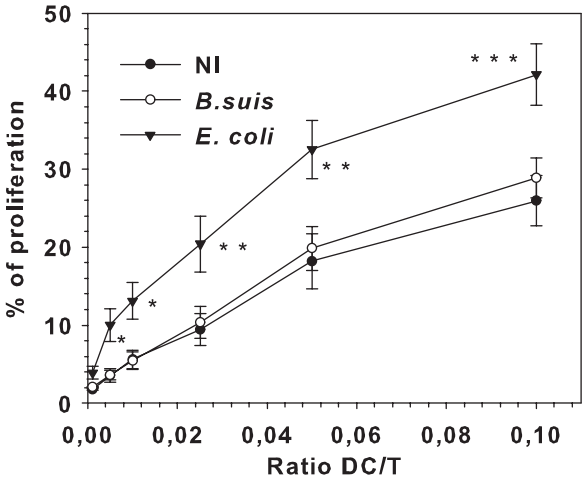

FIG. 2. Ability of infected DCs to induce naïve T-lymphocyte proliferation. At 24 h p.i. DCs that were not infected (NI) or were infected with *B. suis* or *E. coli* were tested for the ability to stimulate allogeneic naïve $CD4^+$ T-lymphocyte proliferation as characterized by CFSE analysis after 5 days of coculture. The data are means ± standard errors of the means of 11 independent experiments. Statistical differences for comparisons with noninfected DCs, as determined by paired Student's *t* tests, are indicated as follows: one asterisk, $P < 0.05$; two asterisks, $P < 0.01$; and three asterisks, $P < 0.001$.

RESULTS

DCs infected with *B. suis* do not engage their maturation process. *Brucella* efficiently infects immature human DCs and is able to grow extensively within these cells (5). In order to determine the consequences for DC physiology, we analyzed the expression of characteristic surface markers on immature and *Brucella*-infected DCs. *E. coli*-infected DCs were used as a positive control, since this bacterium induces strong maturation of DCs (47). *Brucella*-infected cells maintained a DC phenotype, as shown by the stable level of expression of DC-SIGN and CD1a (Fig. 1) and the absence of CD14 expression, indicating that the bacteria did not cause the DCs to retreat towards a monocyte/macrophage phenotype. Evident up-regulation of membrane determinants involved in antigen presentation (major histocompatibility complex classes I and II) and costimulation (CD83, CD40, and CD86) were observed in *E. coli*-infected DCs. In contrast, in *Brucell*a-infected DCs, the expression of these maturation markers was weakly modulated compared to the expression in immature DCs. The slight increase in CCR7 and CD83 expression meant that infection with *Brucella* resulted in very limited activation of DCs, which did not lead to cell maturation. Repeated experiments with DCs from several donors ($n = 22$) confirmed that *Brucella*-infected cells did not exhibit increased expression of the chemokine receptor CCR7, of the antigen presentation molecules HLA-ABC and HLA-D, and of the costimulation receptors CD40, CD86, and CD83 ($P < 0.001$ for these six markers compared to *E. coli*-infected DCs). Taken together, these results showed that, in spite of a high infection rate and extensive proliferation within DCs (involving close contact with a great number of bacteria) (5), *Brucella* did not induce up-regulation of maturation marker expression in human DCs.

***Brucella*-infected DCs are poor inducers of human naïve T-lymphocyte proliferation.** The functional impact of the infection was assessed by analyzing antigen presentation activity of *Brucella*-infected DCs to naïve T cells. Immature DCs were infected with *Brucella* and *E. coli* as a positive control for antigen presentation by fully mature DCs. After 24 h of infection, DCs were put in contact with allogeneic human naïve $CD45RA^+$ $CD4^+$ T cells intracellularly stained with CFSE and plated at DC/T-cell ratios varying from 0.1 to 0.005. T-lymphocyte proliferation was determined 5 days later by flow cytometry analysis of the decrease in CFSE (Fig. 2). DCs infected with *E. coli* clearly showed a higher capacity to induce the response of naïve T cells than immature resting DCs. In contrast, *Brucella*-infected DCs were unable to provide more efficient stimulation of T-cell proliferation than immature DCs, and there was no statistical difference between the antigen-presenting cell (APC) activity of immature DCs and that of *Brucella*-infected DCs. This indicated that *Brucella* was also able to circumvent DC maturation at the functional level.

DCs infected with *Brucella* are poor producers of TNF-α. TNF-α is a multipotent inflammatory cytokine fundamental for defense against a variety of intracellular pathogens and plays an important role in DC maturation (44). Moreover, we showed several years ago that *B. suis* specifically prevents human macrophages from secreting TNF-α (9). Therefore, we measured TNF-α secretion by human DCs infected with *Brucella* or *E. coli* (Fig. 3). In contrast to the results obtained with *E. coli*, which induced remarkable secretion of TNF-α, DC invasion by *Brucella* did not promote any comparable production of this cytokine. The concentrations of TNF-α in *Brucella*-infected DC supernatants remained very low and were just above those observed in immature DC supernatants. The absence of IL-10 production by infected DCs (data not shown) suggested that *Brucella* could affect the production of TNF-α in an IL-10-independent way.

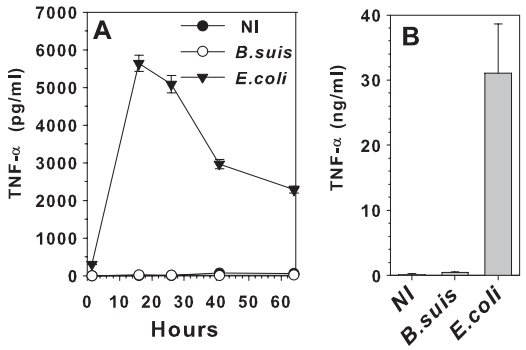

FIG. 3. TNF-α secretion during human DC infection. DCs were infected with *B. suis* or *E. coli* or were not infected (NI) as described in the text. Supernatants were assayed to determine TNF-α concentrations. (A) Change in TNF-α concentration during DC infection (means ± standard errors of the means of triplicate determinations; one experiment representative of four experiments). (B) Concentrations of TNF-α at 24 h p.i. (means ± standard errors of the means of 23 independent experiments).

Exogenous TNF-α promotes the maturation of *Brucella*-infected DCs and antigen presentation. Addition of *E. coli* lipopolysaccharide (LPS) to *Brucella*-infected DCs led to complete phenotypic maturation of these cells (data not shown). This result implied that the lack of DC maturation during *Brucella* infection did not result from a total blockade of their engagement in maturation processes but more likely resulted from impairment of one or several parameters controlling these processes. Given that TNF-α is strongly implicated in DC maturation and that TNF-α knockout DCs fail to mature (44), the impairment of TNF-α secretion during DC infection could be a pertinent way for *Brucella* to avoid the maturation of infected cells. We analyzed the behavior of *Brucella*-infected DCs in the presence of exogenous TNF-α. DCs were infected with the bacteria, rhTNF-α was added at 16 h p.i. (the time of peak TNF-α secretion observed in *E. coli*-infected DCs [Fig. 3A]), and DC maturation was analyzed at 48 h p.i. (Fig. 4). DCs infected with *Brucella* and treated with exogenous TNF-α displayed highly significant enhancement of CCR7 and CD83 expression compared to untreated infected cells. Similarly, the expression levels of CD40, CD86, HLA-ABC, and HLA-D were up-regulated in the presence of TNF-α. Exogenous TNF-α did not increase expression of the maturation markers to the levels resulting from infection with *E. coli*. Nevertheless, TNF-α treatment undoubtedly promoted the initiation of maturation processes in *Brucella*-infected DCs.

In the presence of exogenous rhTNF-α, *Brucella*-infected DCs acquired the ability to induce powerful proliferation of naïve T cells that was quantitatively similar to that induced by fully mature *E. coli*-infected DCs (Fig. 5). These results indi-

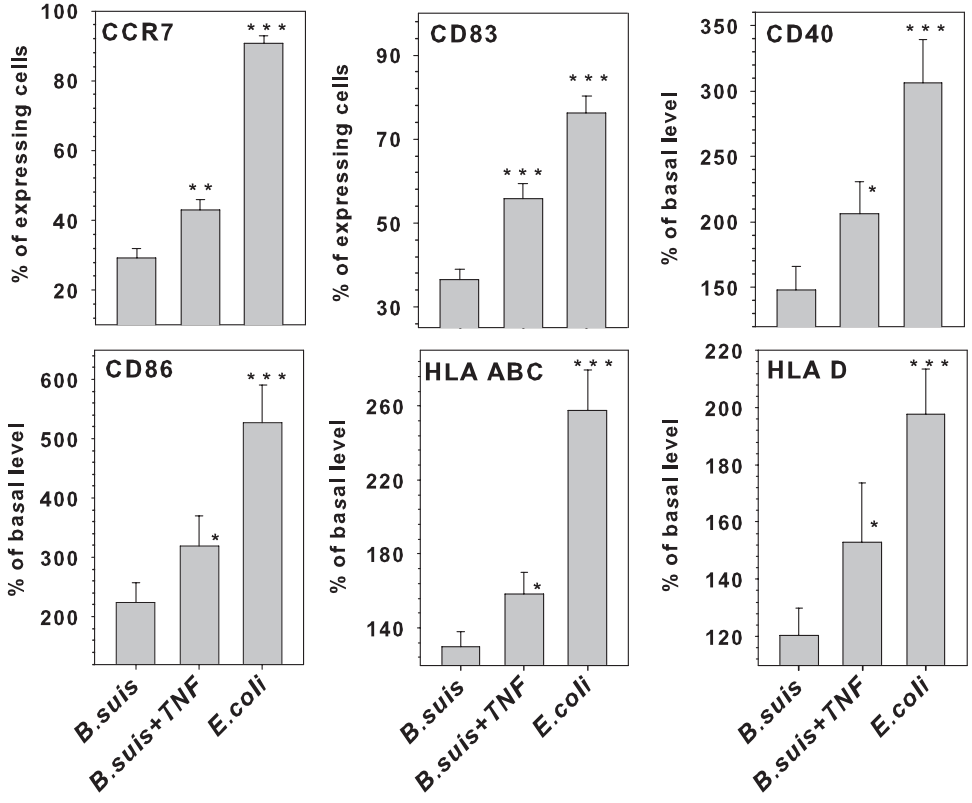

FIG. 4. Effect of exogenous rhTNF-α on *Brucella*-infected DC phenotype. DCs were infected with *Brucella* or *E. coli*. At 16 h p.i., 10 ng/ml of rhTNF-α was added when needed (*B.suis*+TNF). Maturation marker expression was analyzed at 48 h p.i. The bars indicate the percentages of expressing cells or up-regulation of fluorescence intensity compared to the basal level measured for immature uninfected cells (means ± standard errors of the means of 13 independent experiments). Statistical differences for comparisons with untreated *Brucella*-infected DC are indicated as follows: one asterisk, $P < 0.05$; two asterisks, $P < 0.01$; and three asterisks, $P < 0.001$. The statistical differences were computed by paired Student's *t* tests for CD40, CD86, HLA-D, and HLA-ABC and by Wilcoxon rank tests for CCR7 and CD83.

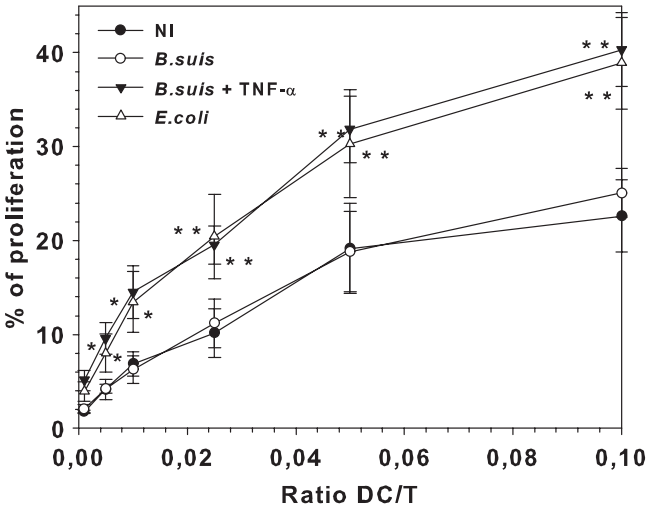

FIG. 5. Effect of exogenous TNF-α on T-cell priming by *Brucella*-infected DCs. DCs were infected with *Brucella* or *E. coli* or were not infected (NI). At 16 h p.i., 10 ng/ml of rhTNF-α was added when needed (*B.suis* + TNF) and maintained throughout the experiment. At 24 h p.i. the cells were tested at different DC/T-cell ratios for stimulation of allogeneic naïve $CD4^+$ T cells. After 5 days, lymphoproliferation was analyzed (means ± standard errors of the means of six independent experiments). Statistical differences for comparisons with immature uninfected DCs, as determined by paired Student's *t* tests, are indicated as follows: one asterisk, $P < 0.05$; and two asterisks, $P < 0.01$.

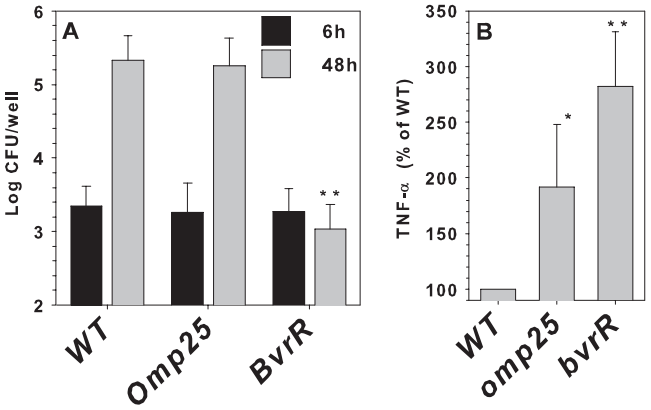

FIG. 6. Intracellular survival of *B. suis omp25* and *bvrR* mutants and induction of cytokine secretion in human DCs. (A) DCs were infected with *B. suis* WT or the *omp25* or *bvrR* mutant. At 6 and 48 h p.i., the numbers of viable intracellular bacteria were determined (means ± standard errors of the means from three independent experiments). (B) Supernatants obtained at 24 h p.i were assayed to determine TNF-α concentrations. The results represent up-regulation of cytokine secretion (means ± standard errors of the means of eight independent experiments) compared to WT-infected DC secretion. Statistical differences for comparisons with WT-infected DC, as determined by paired Student's *t* tests (A) or by Wilcoxon rank tests (B), are indicated as follows: one asterisk, $P < 0.05$; and two asterisks, $P < 0.01$.

cated that the impairment of TNF-α production observed upon *Brucella* infection could account for the restriction of DC maturation and for the absence of antigen presentation to naïve T lymphocytes.

The results described above also suggest that the amount of TNF-α produced upon *E. coli* infection was much greater than the amount required for the establishment of optimal APC activities. This was confirmed by biological assays (9); the TNF-α biological activity measured in culture supernatants of *E. coli*-infected DCs was at least 10-fold higher than the rhTNF-α biological activity allowing optimal APC activity of infected DCs (data not shown).

***omp25* and *bvrR* mutants of *B. suis* have reduced ability to control TNF-α secretion during human DC infection.** Omp25 is an outer membrane protein present on the surface of virulent *Brucella*, and Omp25-defective mutants are attenuated in vivo (14). Some years ago we demonstrated that this protein promoted the inhibition of TNF-α production following human macrophage infection with *Brucella* or stimulation of human macrophages by *E. coli* LPS (9, 27). Omp25 is also absent from the external membrane of *Brucella* with a mutation in the *bvr* operon (22), since the BvrR/S two-component system regulates the expression of the *omp25* gene. *bvrR* mutants of *Brucella* are attenuated in mice and proliferate neither in isolated macrophages (30, 52) nor in DCs (5). These findings prompted us to analyze the interactions of two mutants with human DCs in order to explore the relationship between the control of TNF-α secretion and the maturation of infected DCs.

Figure 6A shows the behavior of *B. suis bvrR* and *omp25* mutants within DCs. At the onset of the experiments, the numbers of intracellular viable bacteria were similar for all bacterial strains. In accordance with our previous findings (5), the *bvrR* mutant did not proliferate within DCs. In contrast, the *omp25* mutant did not display any significant impairment of intracellular proliferation compared to the WT, a result similar to that obtained in vitro with human macrophages (27).

When cytokine production was examined, human DCs infected with the *omp25* or *bvrR* mutant produced more TNF-α than WT-infected DCs (for the WT-*omp25* mutant comparison, $P = 0.0363$; for the WT-*bvrR* mutant comparison, $P = 0.0014$) (Fig. 6B), and the secretion was more pronounced with the *B. suis bvrR* mutant. The secretion of TNF-α induced by the *omp25* mutant was similar to that previously measured with human macrophages (27). These findings indicated that, as in macrophages, Omp25 did not contribute to the intracellular proliferation of the bacteria but had an important role in the control of TNF-α production by infected DCs, with possible consequences for DC maturation.

DCs infected with *B. suis* mutants mature and stimulate naïve T lymphocytes by a TNF-α-dependent mechanism. Figure 7 shows the maturation of DCs infected with the *omp25* and *bvrR* mutants of *B. suis*. The percentages of DCs expressing CCR7 and CD83 were higher in DCs infected with the mutants than in WT-infected cells. In the same way, the expression levels of CD40, CD86, and HLA-ABC were higher when infections were performed with mutants than when infections were carried out with the WT strain. This indicated that DCs infected with the *omp25* or *bvrR* mutant of *B. suis* displayed significant phenotypic maturation compared to WT-infected cells.

However, the maturation level reached upon infection with the *omp25* mutant was not equivalent to that observed in *bvrR* mutant-infected cells. Although the expression of CD40, CD86, and HLA-ABC was modulated equivalently in DCs infected with the two mutants, HLA-D expression was significantly induced in *bvrR* mutant-infected DCs ($P = 0.03$ for a

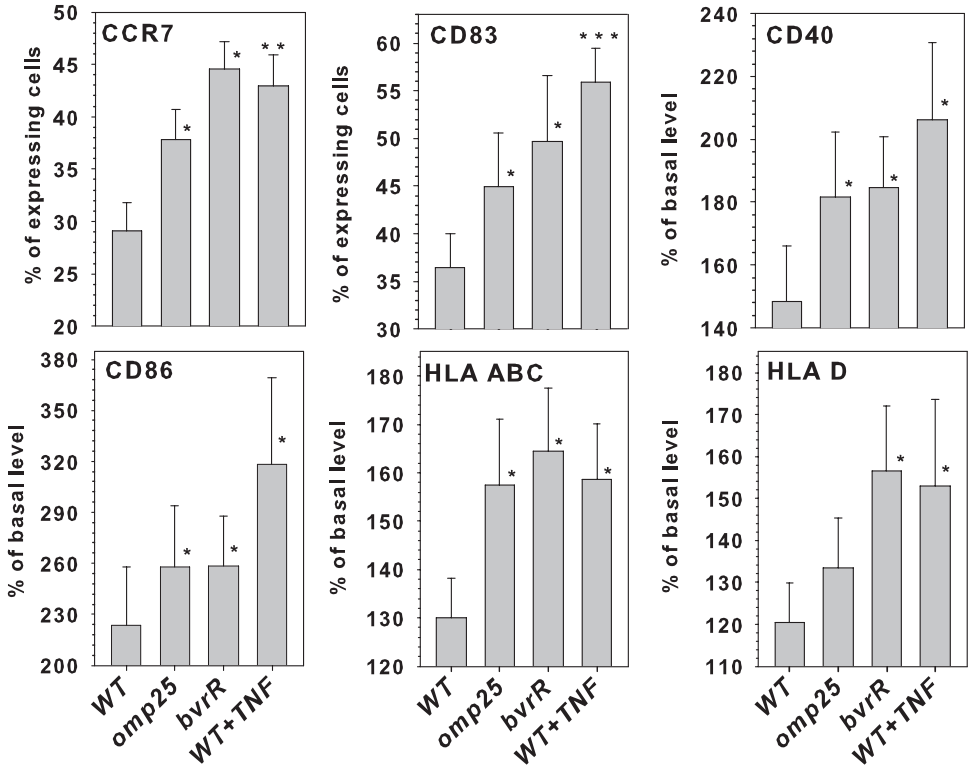

FIG. 7. Analysis of DC maturation after infection by *Brucella* mutants. DCs were infected with the *B. suis* WT (WT and WT+TNF), *omp25* mutant, or *bvrR* mutant. At 16 h p.i., 10 ng/ml of rhTNF-α was added when needed (WT+TNF). Maturation marker expression was analyzed at 48 h p.i. The bars indicate the percentages of expressing cells or up-regulation of fluorescence intensity compared to the basal level measured with immature uninfected cells (means ± standard errors of the means from 10 independent experiments). Statistical differences for comparisons with untreated WT-infected DC are indicated as follows: one asterisk, $P < 0.05$; two asterisks, $P < 0.01$; and three asterisks, $P < 0.001$. The statistical differences were computed by paired Student's *t* tests for CD40, CD86, HLA-D, and HLA-ABC and by Wilcoxon rank tests for CCR7 and CD83.

comparison with the WT) but not in *B. suis omp25* mutant-infected DCs despite a slight increase ($P = 0.445$ for a comparison with the WT). Moreover, CCR7 and CD83 were more strongly expressed in *B. suis bvrR* mutant-infected DCs than in *B. suis omp25* mutant-infected cells (for CCR7, $P = 0.005$, for CD83, $P = 0.039$). This meant that the commitment to maturation processes induced with the *B. suis bvrR* mutant was deeper than the commitment induced with the *omp25* mutant.

For each of the two mutants, the expression of CCR7 and HLA-ABC was equivalent to that measured in *B. suis*-infected cells treated with rhTNF-α (WT plus TNF-α [Fig. 7]). The same observation was made for HLA-D expression on *bvrR* mutant-infected DCs (but not on *omp25* mutant-infected DCs). The CD83 and CD40 expression levels appeared to be slightly lower but still close to those measured in *B. suis*-infected DCs treated with rhTNF-α. Only CD86 expression was less potently up-regulated in mutant-infected cells than in WT-infected rhTNF-α-treated DCs.

This phenotypic analysis was confirmed by assessment of the stimulating capacities of mutant-infected DCs. Figure 8A shows the proliferation of naïve T cells cultured for 5 days in the presence of DCs infected with the *B. suis omp25* or *bvrR* mutant at a DC/T-cell ratio of 0.025 (1:40). Upon infection with these mutants, DCs acquired the capacity to efficiently present antigen to naïve T cells. Again, the effect was more pronounced with the *bvrR* mutant than with the *omp25* mutant ($P = 0.01$); the former displayed approximately 80% of the stimulation obtained with *E. coli*, whereas the latter displayed only about 50% of the full-stimulation effect.

Thus, upon infection by Omp25-defective mutants, DCs matured and acquired the ability to present antigens, and these effects were stronger when the lack of Omp25 protein was associated with impairment of the BvrR/S system. In order to determine whether TNF-α secreted during DC infection with mutants is responsible for the induction of DC maturation, the experiments described above were repeated in the presence of anti-TNF-α blocking antibodies. When these antibodies were added at the onset of infection, the *omp25* and *bvrR* mutants were unable to induce an increase in maturation marker expression (data not shown). In parallel, mutant-infected DCs failed to stimulate naïve T cells. Figure 8B shows the proliferation of naïve T cells cultured with *omp25* or *bvrR* mutant-infected DCs with or without anti-TNF-α blocking antibodies. For each mutant, the inhibition of TNF-α biological activity led to a complete collapse of antigen presentation activities ($P < 0.001$ for both mutants), which fell to the basal level measured in DCs infected with virulent *Brucella* or in immature uninfected DCs (Fig. 8B).

IL-12 secretion by *Brucella*-infected human DCs. IL-12 production by DCs is essential to drive a Th1 immune response, which is important for the clearance of intracellular pathogens (46). In order to determine whether the infection can influence

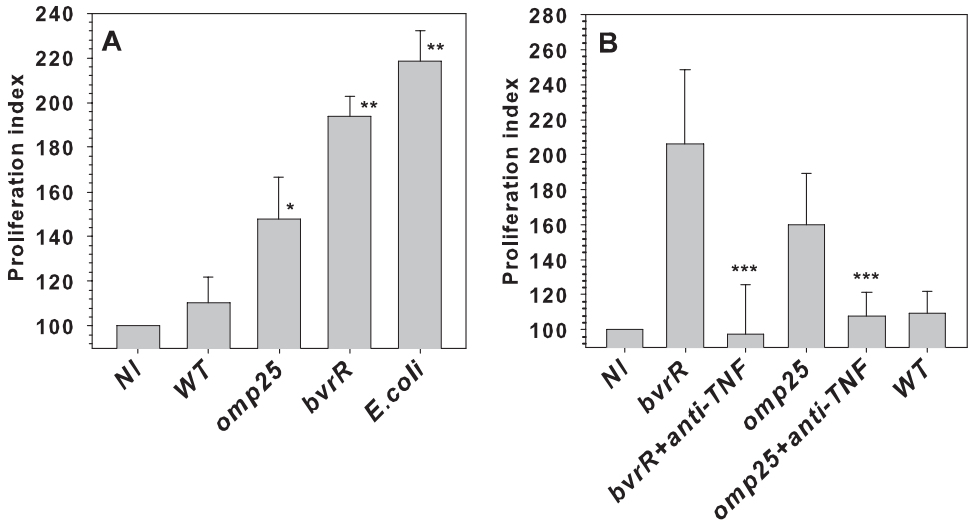

FIG. 8. Induction of naïve T-cell proliferation by mutant-infected DCs. (A) DCs were infected with the *Brucella* WT, *omp25* mutant, or *bvrR* mutant or *E. coli.* At 24 h p.i., cells were tested for stimulation of allogeneic naïve $CD4^+$ T cells (DC/T-cell ratio, 1:40). T-lymphocyte proliferation was analyzed 5 days later, and the proliferation index was calculated by comparison with noninfected cells (NI) (means ± standard errors of the means of seven independent experiments). (B) Same experiments described above for panel A but anti-TNF-α antibody was added at 1 h p.i. where indicated and maintained throughout the experiment (means ± standard errors of the means of five independent experiments). Statistical differences were calculated by comparison with immature uninfected DCs (A) or by comparison with the corresponding condition without blocking antibody (B) by using paired Student's *t* tests or Wilcoxon rank tests (one asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$).

the secretion of cytokines involved in the polarization of the immune response, we measured IL-12 concentrations in culture supernatants of DCs infected with WT or attenuated strains (Fig. 9). DCs infected with virulent *B. suis* produced smaller amounts of IL-12 than cells infected with the *omp25* or *bvrR* mutant ($P < 0.05$ and $P < 0.01$, respectively, calculated from seven independent experiments). Infection with the *bvrR* mutant of *B. suis* led to more intense IL-12 secretion ($P = 0.031$ for a comparison of the *bvrR* and *omp25* mutants [$n = 7$]). These results suggested that infection with these mutants not only induced DC maturation but could also favor Th1 polarization of the adaptive response.

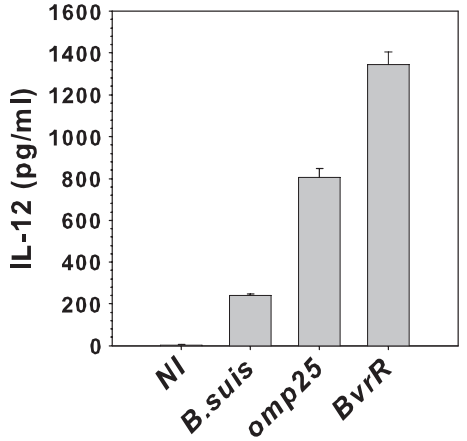

FIG. 9. IL-12 secretion by human DCs infected with *Brucella* WT or mutants. DCs were infected with the *Brucella* WT, *omp25* mutant, or *bvrR* mutant or were not infected (NI). At 24 h p.i., culture supernatants were assayed to determine IL-12 concentrations. The data (means ± standard errors of the means) are from one experiment representative of eight independent experiments.

DISCUSSION

We have recently established that, unlike most intracellular bacteria, *Brucella* invades human DCs and grows extensively within them. In order to assess whether this behavior of *Brucella* specifically alters DC functions, we analyzed the consequences of *Brucella* infection for DC physiology and characterized the implications of some virulence factors.

Unlike monocyte-derived DCs infected with *Mycobacterium* (35), *Brucella*-infected DCs retained a DC phenotype, as shown by DC-SIGN expression and the absence of CD14. A dedifferentiation of infected DCs towards a monocyte-macrophage phenotype would obviously have had dramatic consequences for immune response initiation. However, even though the differentiation state of human DCs was not modified, infection with *Brucella* did not trigger DC maturation, as demonstrated by the low level of expression of adequate markers on the cell surface and by the inability of infected cells to present antigen to naïve T cells. A comparable inhibition of DC maturation was reported previously after in vitro infection with various intracellular pathogens, notably several viruses, including herpes simplex virus, hepatitis C virus, vaccinia virus, human T-cell leukemia virus, and measles virus (33), and also intracellular parasites, including *Toxoplasma* and *Trypanosoma* (45). In contrast, the majority of intracellular bacteria induce DC maturation during their interaction with these cells; this is particularly true for *Bordetella* (16), *Listeria* (42), *Chlamydia* (40), and *Salmonella* (38, 53). For *Mycobacterium tuberculosis*, although a study reported inhibition of *Mycobacterium*-infected DC maturation (23), several other workers have observed maturation of human and murine infected DCs (11, 24). Fewer reports are available about the interaction between *Legionella* and DCs, and the induction of DC maturation by these bacteria still seems to be debated (28, 36, 37). Of the two

bacteria which, like *Brucella*, proliferate in human DCs, *Francisella* apparently fails to control maturation and seems to make DCs develop towards an aberrant activation state (4, 6), whereas *Coxiella* manages to avoid DC maturation owing to its low endotoxic properties (48). *Brucella* compounds are also reported to be weakly endotoxic and participate in the stealthiness of the bacteria (25, 32). However, the low stimulatory activity of *Brucella* cannot explain by itself the lack of DC maturation since Omp25-defective bacteria did induce significant maturation in spite of their endotoxic activity comparable to that of WT *Brucella* (34). Therefore, the unusual absence of DC maturation during interaction with WT *Brucella* involves mechanisms in which TNF-α seems to play a key role: (i) TNF-α, which is an essential factor for DC maturation (44, 54), was not produced during DC infection; (ii) TNF-α addition to the infected DCs overcame the lack of both phenotypic and functional maturation of the cells; (iii) mutant-infected DCs, which produced TNF-α, displayed characteristics of maturation; and (iv) blocking anti-TNF-α antibodies prevented the maturation of *omp25* and *bvrR* mutant-infected DCs. Our results thus define an original virulence strategy by which *Brucella* could manipulate the specific immune response by targeting DC functions.

In contrast to WT *Brucella*, the two Omp25-defective mutants induced TNF-α production by infected DCs. This observation agrees with previous data obtained with macrophages (27) and suggests that TNF-α secretion is probably similarly regulated in human DCs and macrophages. It also indicates that impairment of TNF-α production is not required for the intracellular development of *Brucella*. Therefore, the decrease in TNF-α production is relevant only to the developing immune response and inflammation.

As previously reported for macrophages, the Omp25 protein does not definitively block TNF-α secretion, which remains inducible (9, 27). *Brucella* is thus able to prevent DC maturation but does not strictly impair it, as shown by treatment of infected cells with rhTNF-α (or with *E. coli* LPS). However, whether the low level of TNF-α secretion is the only parameter accounting for the weak maturation of infected DCs remains unclear; we cannot prejudge eventual Omp25-mediated complementary mechanisms not related to TNF-α control that could also down-regulate infected DC maturation. Nevertheless, the abrogation of TNF-α biological activity with blocking antibodies is sufficient to suppress the maturation of mutant-infected DCs, proving the key role of this cytokine in the maturation avoidance strategy.

The possible contribution of complementary mechanisms is supported by the less intense maturation of *omp25* mutant-infected DCs than of *bvrR* mutant-infected DCs. This could result directly from the very different fates of the two mutants within their host cells. Actually, the intracellular proliferative capacity of the *B. suis omp25* mutant could allow persistent virulence processes, while the elimination of the intracellular *bvr* mutant could lead to more potent stimulation of DCs by degraded bacterial compounds. We cannot exclude the possible direct implication of other bacterial factors regulated by the BvrR/S two-component system. Indeed, *bvrR* invalidation alters the expression of more than 100 proteins of *Brucella*, including Omp3 family proteins, peptidoglycan-related proteins, and several lipoproteins (31). Moreover, if lipid A underacylation in the *bvrR* mutant does not affect LPS endotoxic activities (34), it could lead to exposition of molecules then recognized by DCs. All of these phenomena could act together with Omp25 to strengthen the control of TNF-α secretion, explaining the larger amount of this cytokine produced upon infection with bacteria lacking both the BvrR and Omp25 proteins than upon infection with the *omp25* single mutant. These factors could play a role in another mechanism implicated in the restraining of DC maturation complementary to the main strategy described here involving the control of TNF-α secretion.

Until now, the drastic attenuation of Omp25-defective *Brucella* in vivo was attributed to the involvement of this protein in the impairment of TNF-α secretion in macrophages (27), whereas the attenuation of *bvr* mutants was related to their inability to proliferate within macrophages (52). Indeed, we show here that, due to their inability to control TNF-α secretion, Omp25-defective bacteria fail to dampen DC maturation and consequently authorize the establishment of a specific immune response, independently of bacterial intracellular proliferation. All of these results could explain why Omp25-defective mutants are attenuated in vivo and why they have been reported to induce protection at least equivalent to that induced by reference vaccine strains (14). In a vaccinal context, the weaker capacity of *omp25* mutants to induce adaptive immunity could be compensated for by the duration of maturation signals resulting from their efficient proliferation during the first phase of host infection (14, 15).

Persistent infection with *Brucella* is associated with a decrease in the capacity for T-lymphocyte proliferation (19) and with down-activation of $CD4^+$ T cells, particularly during relapses of chronic infection (50). Antibiotic treatment leads to normalization of these parameters, confirming the connection between the persistence of living bacteria within patients and the reported defect in a T-cell response. Thus, an association can be proposed between the impairment of maturation and APC activities of DCs by *Brucella*, and the immunological status of brucellosis patients. A Th2-orientated response is usually observed in chronic brucellosis patients (19, 43), whereas protection against *Brucella* is conferred by a Th1-polarized response. Myeloid DCs have been implicated in the induction of Th1 responses, particularly through IL-12 secretion. The absence of both IL-12 production and T-cell stimulatory activity in *Brucella*-infected DCs clearly suggests that by altering and preventing myeloid DC functions *Brucella* specifically avoids the development of a protective Th1 immune response. Moreover, human immature DCs interacting with naïve $CD4^+$ T cells could induce a regulatory T-cell response and inhibit the Th1 response (17). By contact with *Brucella*-infected immature DCs, $CD4^+$ naïve T cells could thus develop in *Brucella*-specific regulatory T cells, which could then delay the adaptive immune response. Furthermore, due to their inefficient interaction with *Brucella*-infected DCs, $CD4^+$ T cells could fail to provide the licensing signals required for subsequent stimulation of $CD8^+$ cytotoxic T cells by infected DCs (51).

A recent publication suggested that a peculiar DC subpopulation, called inflammatory DCs, could participate in the effector phase of the anti-*Brucella* immune response in infected mice (12). Nevertheless, host interactions with *Brucella*, as well

as the subpopulations of DCs, are not fully equivalent in mice and humans. Therefore, it is still possible that such a mechanism cannot be transposable to humans.

To summarize, our data show that *Brucella* avoids maturation and APC activities of human myeloid DCs through Omp25-dependent mechanisms disrupting TNF-α secretion by infected cells. The crucial importance of TNF-α in this bacterial escape strategy enlightens previous reports on the association of chronic *Brucella* infections with an unfavorable TNF-α promoter polymorphism (8, 13). Altogether, the data suggest that some host genetic factors could confer a particular susceptibility to chronic brucellosis and be decisive for spontaneous healing or establishment of chronic disease by impeding the complete eradication of the pathogens. In conclusion, targeting DC maturation through TNF-α regulation could be an essential parameter of the *Brucella* virulence strategy and pathogenicity.

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